

Angiogenesis in autosomal-dominant polycystic kidney disease

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Background. Autosomal-dominant polycystic kidney disease (ADPKD) is a genetic disorder that is responsible for approximately 10% of all cases of end-stage renal disease (ESRD). It is characterized by the formation of epithelial cell cysts, an increase in the extracellular matrix, and vascular alterations believed to be the result of compression by the cysts. Our recent observations demonstrated a rich vascular network on the surface of the cysts, and thus, we postulated that angiogenesis could be a factor in the progression of ADPKD.

Methods. Kidneys removed from patients with ADPKD were studied using (1) angiographs, (2) immunostaining [factor VIII-related antigen, vascular endothelial growth factor (VEGF), VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2), metalloproteinase-2 (MMP-2), and integrin $\alpha_v\beta_3$], and (3) Western blot analysis and enzyme-linked immunosorbent assay. The expression of VEGF₁₆₅ in ADPKD cells in culture was determined.

Results. There was (1) an extensive capillary network in the cyst wall of ADPKD kidneys, (2) morphological evidence of vascular malformations, (3) expression of VEGF₁₆₅ in cyst cells of VEGFR-2 in endothelial cells and an absence of VEGFR-1 in endothelial cells, (4) secretion of VEGF₁₆₅ by ADPKD cyst cells in culture, and (5) coexpression of matrix MMP-2 and integrin $\alpha_v\beta_3$ in vessels from ADPKD.

Conclusions. There is angiogenesis in ADPKD. This process may be necessary for cyst cells to grow and may be responsible for increased vascular permeability facilitating fluid secretion into the cysts. Neovascularization may result in the formation of aneurysms responsible for the renal bleeding in this disease.

Autosomal-dominant polycystic kidney disease (ADPKD) is a frequent genetic disorder responsible for approximately 10% of all cases of end-stage renal disease (ESRD) [1–3]. Little information exists on the distribution and characteristics of the microcirculation of the kidney in ADPKD. Before the advent of renal ultrasound, computed tomography scans and magnetic reso-

nance imaging angiographic studies were utilized to diagnose ADPKD, and revealed separated and distorted large vessels displaced by the cyst growth. Using carmine-colored gelatin, in 1929 Ritter and Baehr described that the “*interlobar and interlobular arteries . . . [in ADPKD] largely lie in the cyst walls . . . [and that] in almost every one of the larger cysts there are numerous arteries of various sizes which lie directly beneath the lining epithelium*” [4–7]. Compression of the tubules and vasculature by the cysts and effects of pressor substances have been suspected to participate in the progression to end-stage renal disease (ESRD) [8, 9]. Abnormalities in the anatomy of the renin-secreting cells were reported by Graham and Lindop, who described that “*renin containing cells were . . . found in attenuated arteries in the wall of cysts*” and that “*about half of the cells containing renin are situated in arteries*” [10].

Inasmuch as the disease is characterized by the proliferation of poorly differentiated epithelial cells and the increase of the extracellular matrix, it seems obvious that the blood supply needs to be adequate to provide oxygenation and nutrients in proportion to the expansion of the epithelium and interstitium. Early observations in our laboratory indicated the existence of a rich vascular network on the surface of cysts from ADPKD. We postulated that angiogenesis could be a factor in the progression of ADPKD. Whereas angiogenesis is fundamental to the processes of reproduction, development and repair [11, 12], it also plays a role in pathological processes, including cancer. Angiogenesis is stimulated by several growth factors, including vascular endothelial growth factor (VEGF) [13–16]. Early in the process of angiogenesis, the endothelial cell basement membrane and surrounding collagen and other matrix proteins are degraded by matrix metalloproteinases, and the capillary basement membrane is disrupted, allowing migration of the endothelial cells from preexisting vessels and subsequent proliferation [13, 17]. Metalloproteinases bind to cell adhesion molecules such as integrin $\alpha_v\beta_3$, which are induced by angiogenic factors. In the normal adult kid-

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ney, constitutive expression of VEGF and VEGF receptors is confined to the glomerulus (podocytes and capillaries, respectively) and some collecting ducts and peritubular capillaries [18].

Our current study reports (1) the existence of an extensive capillary network in the cyst wall of ADPKD kidneys removed from patients in renal failure (ESRD), (2) morphological evidence for vascular malformation, (3) the expression of the VEGF in cyst cells and of VEGF receptor-2 in cyst cells and vessels, (4) the secretion of VEGF by ADPKD cells in culture, and (5) the coexpression of matrix metalloproteinase-2 (MMP-2) and of integrin $\alpha_v\beta_3$ in vessels from ADPKD. Taken together, these findings are indicative of angiogenesis in ADPKD, a process that may be necessary for cyst cells to grow as well as being responsible for increased vascular permeability—facilitating fluid secretion into the cysts—and a likely cause of the formation of aneurysms and renal bleeding found in this disease.

METHODS

Ten kidneys surgically removed because of medical indications from patients with ESRD secondary to ADPKD, donated for research to the Polycystic Kidney Research Foundation, were used. Four additional kidney preparations were available from the archives of the Department of Pathology, and new sections were obtained for light microscopy and immunostaining. The mean age (\pm SD) of the patients at the time of kidney removal was 55.2 ± 4.1 years. Of the 14 patients (6 males and 8 females), 12 were white and 2 were Hispanic. Nine were on dialysis at the time of transplant, and five had a previous transplant and were receiving immunosuppression. Two patients had hypertension. The kidneys were removed for medical reasons; the investigators were not involved in the decision making conducive to surgery.

Some kidneys were perfused immediately after removal with lactate Ringer's solution containing heparin or with cold storage solution (ViaSpan®; DuPont, Merck Pharmaceutical Co., Wilmington, DE, USA) and delivered to our laboratory in wet ice. In preliminary experiments, a branch of the renal artery was cannulated and the tissue was perfused with a lactate-buffered physiological solution (pH 7.4, at 4°C to 10°C) also containing 50 g/L of Pentafraction® (DuPont), adenosine 1.3 g/L, allopurinol 0.136 g/L, and glutathione 0.922 g/L. The perfusion rate was ~ 1000 mL/hour, and the venous effluent was discarded. Angiograms were obtained after injection of Hypaque™ and Fluorescein®. For the latter, the kidney was positioned in front of a Topcon-50× retina camera with filters for fluorescence measurements. Images were captured by an OIS digital computer using its proprietary

software (OIS, Sacramento, CA, USA) and stored for differed analysis.

For fresh preparations, cyst walls were cut free, stretched on glass slides, covered with 1 mm cover slips, and observed immediately using a Nikon microscope with $\times 100$ to $\times 200$ magnification. Microphotographs were taken with a Nikon camera.

The appearance of ESRD-ADPKD kidneys was rather uniform. Enlarged kidneys measuring up to 30 by 20 by 18 cm had their surfaces covered by randomly distributed cysts of different sizes that also were present through the mass of the kidney. The cysts contained clear or dark fluid, the latter from past hemorrhages. Randomly taken samples for histopathology were provided by pathologists at the University of Texas Medical Branch and other medical centers. The samples corresponded to areas with moderately sized cysts, and the intervening areas were shown to contain some normal-sized tubules and more frequently small cystic tubules. In those areas, the classic description of interstitial fibrosis and coexistence with some preserved glomeruli was confirmed. Hemorrhagic cysts were not studied.

Samples from different areas of the kidney were frozen embedded in Tissue Freezing Medium™ (TBS™; Triangle Biomedical Sciences, Durham, NC, USA) or fixed in 4% paraformaldehyde and embedded in paraffin ($t < 65^\circ\text{C}$). Sections were placed on poly-L-lysine-treated slides. Paraffin blocks were cut at 3 to 4 μm thickness and dried overnight at 40°C. Paraffin was removed, and the sections were rehydrated. For negative controls, the primary antibody was omitted. Positive controls for factor VIII-related antigen were slides from normal kidney tissue, and for VEGF and receptors, positive controls were slides from breast cancer tissues. Staining for factor VIII-related antigen was performed using mouse anti-human monoclonal antibody (Zymed Laboratories, Inc., South San Francisco, CA, USA) and was prediluted and used as per the manufacturer's recommendations. Sections were selected from hematoxylin and eosin-stained slices. An avidin-biotin peroxidase conjugate protocol was used. The chromogene was diaminobenzidine (DAB). Staining for VEGF protein and VEGF-receptors 1 and 2 (VEGFR-1 or flt-1 and VEGFR-2 or flk1/KDR) was performed in sections contiguous to the ones studied for factor VIII-related antigen. The antibodies used were as follows: anti-VEGF monoclonal antibody (NeoMarkers, Union City, CA, USA), which recognizes human isoforms of VEGF, including soluble proteins; specific anti-human VEGFR-1 monoclonal antibody (Flt-1 receptor), and specific anti-human VEGFR-2 (KDR) monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). The procedure was similar to the one described for studies of factor VIII-related antigen staining. Images from immunocytochemistry were acquired using a Nikon microscope model Eclipse E800 (Nikon, Inc., Melville, NY,

USA) and a digital camera (Nikon Coolpix 950), and were saved and stored. Image processing and analysis was performed on a Dell Optiplex GX1 computer equipped with image processing software (Adobe Photoshop; Adobe Systems, Mountain View, CA, USA) using the UTHSCSA *Image Tool* program (developed at the University of Texas Health Science Center at San Antonio, TX, USA by Wilcox et al and available from the Internet at <http://uthscsa.edu/dig/itdesc.html>) [19]. This process was similar to the reported by others [20]. In three different sections for each condition, the image was divided in quadrants (numbered 1 through 4 and sequentially analyzed: picture 1, quadrant 1; picture 2, quadrant 2, etc.) determining the pixel value of cytoplasm areas (in a scale of 0 to 255) of cyst cells, endothelial cells, or interstitial areas of comparative sizes. Nuclei were excluded from analysis.

To demonstrate colocalization for MMP-2 and integrin, $\alpha_v\beta_3$ monoclonal antibodies against MMP-2 (goat, anti-human) and fluorescein-conjugated integrin $\alpha_v\beta_3$ (mouse, anti-human; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. The secondary antibody was anti-goat TRITC IgG1 (Sigma-Aldrich). Areas of colocalization appear yellow. Fluorescence was assessed with a Nikon microscope Model Eclipse E800 (Nikon Inc.). Images were obtained using a Dage Single one-half inch cooled CCD camera (Advanced Scientific, Chamette, LA, USA) and processed with the Meta-morph Imaging System (Universal Imaging Corp., West Chester, PA, USA).

Cell cultures

Autosomal-dominant polycystic kidney disease cells were isolated from ADPKD cysts from kidneys obtained after nephrectomy and cultured in 5% CO₂/95% air at 37°C in Click-RPMI media [21], buffered with 10 mmol/L N-2-hydroxyethylpiperazine n'-2-ethanesulfonic acid (HEPES), and supplemented with 1% fetal bovine serum (FBS), human transferrin (5 mg/mL), hydrocortisone 50 nmol/L–dexamethasone 5 nmol/L, insulin 5 µg/mL, and triiodothyronine 5 pmol/L (GIBCO BRL®, Life Technologies Inc., Rockville, MD, USA). Subcultures were obtained after confluence and passages 1 through 3 were used. MCF-7 cells (human breast cancer cell line) were grown in improved minimum essential medium (IMEM) Eagle's (Richter's medium) (GIBCO BRL®) also containing 10% FBS and penicillin/streptomycin. Proximal tubule cells, obtained from normal areas of kidneys removed because of cancer, were isolated as previously described [22] and cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM)/F-12 media supplemented with 25 mmol/L HEPES; 21.5 mmol/L H₂CO₃; 4 mmol/L L-glutamine; 10 mg/L insulin, 5.5 mg/L transferrin, 2 mg/L ethanolamine, 5 µg/L sodium selenite

(ITES, Sigma-Aldrich, St. Louis, MO, USA), and non-essential amino acids [22]. Passages 1 and 2 were used.

Western blot analysis of cell culture lysates for detection of VEGF

Cells were lysed in lysis buffer (20 mmol/L Tris, 150 NaCl, 1 mmol/L phenylmethyl sulfonylfluoride (PMSF), and 0.25% NP-40). Equal amounts of protein (200 µg) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4 and 20% Tris-Glycine gels; Novex, San Diego, CA, USA) and subsequently transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech., Piscataway, NJ, USA). Blots were probed with a specific monoclonal antibody recognizing VEGF₁₆₅ [VEGF Ab-4 (BFDD31) 1:200 dilution; NeoMarkers, Inc.], followed by horseradish peroxidase-conjugated anti-mouse IgG antibody diluted 1:4000 (Amersham Pharmacia Biotech.). The detection of specific signals was performed using the chemoluminescent substrate for detection of horseradish peroxidase (SuperSignal® West Pico Chemoluminescence Substrate; Pierce, Rockford, IL, USA). Recombinant VEGF₁₆₅ protein (2.5 ng) served as a positive control (Ab-4; NeoMarkers). Ab-4 shows a single band at ~19 kD. Protein from cultured human proximal tubule cells, expecting to have low or no expression of VEGF, and from MCF-7 cells (human breast cancer cell line) was also used. Band intensities were quantitated by densitometry (ImageQuant; Molecular Dynamics, Sunnyvale, CA, USA).

Anti-VEGF enzyme-linked immunosorbent assay

Vascular endothelial growth factor released to the culture medium was measured using anti-human-VEGF antibody as the capture antibody (AF-293-NA) in combination with a biotinylated VEGF affinity-purified polyclonal detection antibody (BAF293; R&D Systems, Inc., Minneapolis, MN, USA) as per the manufacturer's instructions. ADPKD cells and MCF-7 cells (human breast cancer cell line) were grown in 25 cm² culture flasks (Biocoat®; Becton Dickinson Labware, Bedford, MA, USA) to confluence. The medium was removed. Cells were washed three times with PBS and incubated for eight hours in culture medium, as described previously in this article. Some ADPKD cells were incubated for 8 or 72 hours in an atmosphere of 90% N₂/5% O₂/5% CO₂. At the end of the incubation period, the conditioned media were removed, centrifuged at 3000 r.p.m. for 10 minutes, and stored at -20°C until the time of analysis. Triplicates samples were analyzed. The VEGF released was expressed per mg of cell protein. VEGF was not detectable in the culture medium used.

Protein measurements

Protein was measured by colorimetry using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA,

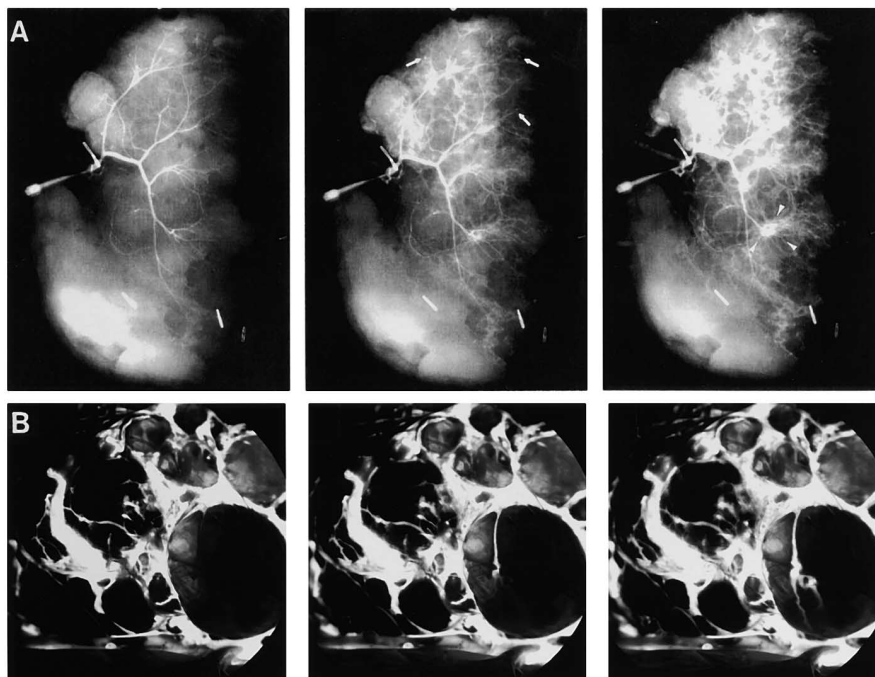


Fig. 1. (A) Radiopaque medium angiogram in a ADPKD kidney perfused in vitro (4°C, with preservation solution at 1000 mL/h) 15 (left panel), 30 (center panel), and 60 seconds (right panel) after bolus injection of 50 mL of Hypaque 60™ in the upper pole renal artery. (Left panel) Note the stretching and displacement of large vessels. (Central panel) Note the vascularization around the cysts and filling of aneurysmatic dilatations (arrows). (Right panel) Note the tuft-like vessels with peripherally broader branches, rather than the normal tapering (arrows). (B) Fluorescent angiogram of the surface of the same kidney (perfused as described for A) injected with 1 mL of Fluorescein® shows vascularization around the cysts, abnormal vessels, and filling of lacunar spaces.

USA) adapted to cuvettes. Bovine serum albumin (BSA) was used as the standard. Absorbance was measured at 750 nm using a Beckman DU-62 spectrophotometer (Beckman, Fullerton, CA, USA).

Statistics

Results are reported as mean \pm SEM unless otherwise stated. Data were analyzed using Student *t* test for unpaired data or population analysis, as required by the experimental design. $P < 0.05$ was considered significant [23].

RESULTS

Angiography suggests angiogenesis around the cysts

Angiography performed in a nephrectomized ADPKD kidney demonstrated the presence of aneurysms and of broad tuft-like vessels as well as extravasation of the contrast medium¹ (Fig. 1A) [24]. In the same kidney, fluorescein was injected in the upper pole artery, and an angiogram was performed. As shown in Figure 1B, there is a rich network of vessels in the cyst wall. The vasculature of the cyst wall includes spiral shaped, tortuous, and dilated vessels. These findings, similar to the ones reported in the diabetic retina [25], are suggestive of angiogenesis.

¹ Angiographs showed extravasation of the contrast, but this observation has to be taken with caution since the kidney studied was subjected to cold ischemia and perfusion with preservation solution, factors that have been reported to alter the vascular permeability.

Small-vessel network found in the walls of ADPKD cysts

As shown in Figure 2 (insert), the cyst walls are highly vascular with randomly distributed tortuous vessels. Microscopic examination of tissues stained for factor VIII-related antigen demonstrated that the cyst walls contained fibrovascular connective tissue. There was intense proliferation of vasculature, predominantly composed of capillaries of varying caliber. In some places, they assumed angiomatous configurations, resembling cavernous angiomata (Fig. 2A). The increased vascularity was also appreciated in the renal interstitial parenchyma away from the cysts, apparently indicating angiomatous formations involving the entire kidney in ADPKD (Fig. 2A). Similar architecture and distribution were found in all ADPKD specimens studied.

In other areas, in the interstitium (Fig. 2B), and especially beneath the cyst epithelium (Fig. 2C), the vessels assumed a “corkscrew” pattern. Ingrowths of neovascularization were also seen into the *lamina propria* of the intraluminal papilliferous projections of the cyst epithelium (Fig. 2D). By comparison, in the normal kidney, the arterial circulation had a quite regular pattern showing a progressive tapering of the caliber of the arterial vessels toward the kidney periphery. The network of capillaries was in close contact with the epithelial basement membrane and also exhibited a notable regularity in diameter at any given cross section of the kidney [26].

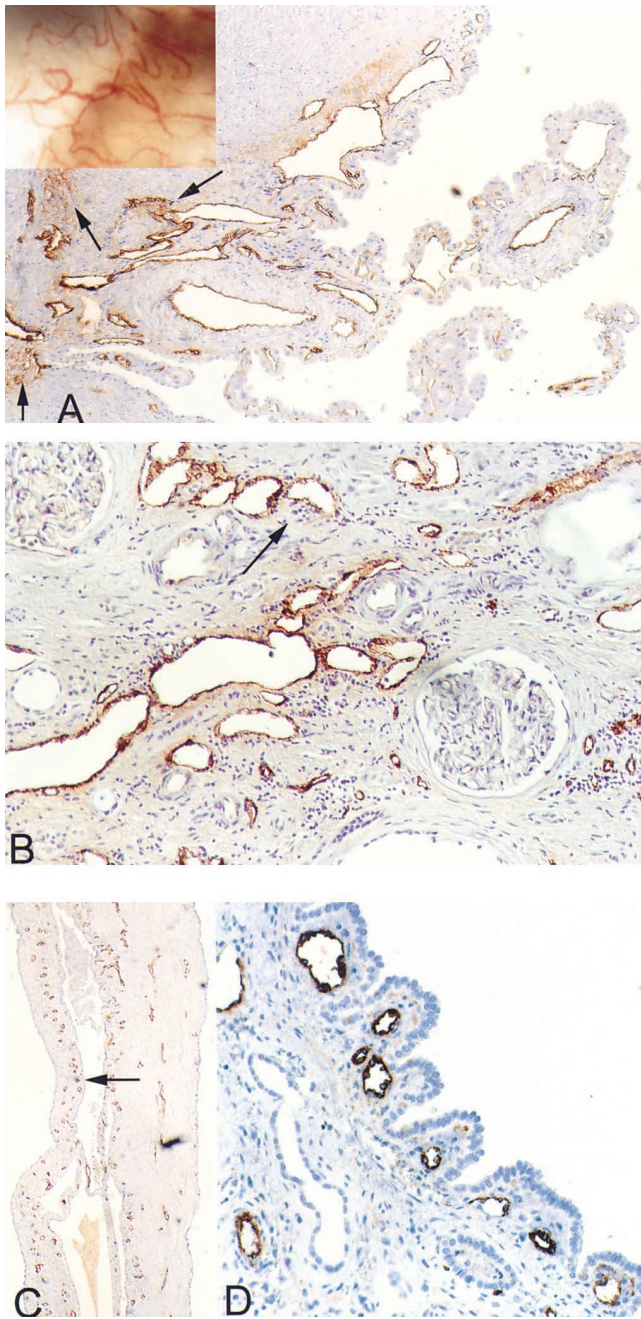


Fig. 2. Vascular network in the wall of ADPKD cysts. (Insert) Freshly mounted cyst wall without staining observed in an inverted Nikon microscope ($\times 200$). Note the capillaries and small vessels containing red cells. (A) Microscopic examination of ADPKD kidney sections stained for factor VIII-related antigen. The cyst walls are composed of fibrovascular connective tissue. There are numerous of capillaries of variable caliber, some of them assuming angiomatous configurations resembling cavernous angiomata (arrows, Nikon $\times 100$). (B) Interstitial area demonstrating dilated vessels in a "corkscrew" pattern. Nikon $\times 200$. (C) Microcirculation beneath a renal cyst shows the "corkscrew" characteristic vasculature seen also in interstitial areas (Nikon $\times 40$). (D) Factor VIII-related antigen staining demonstrating ingrowth of vasculature into the *lamina propria* of the intraluminal papilliferous projections of the cyst epithelium (Nikon $\times 400$).

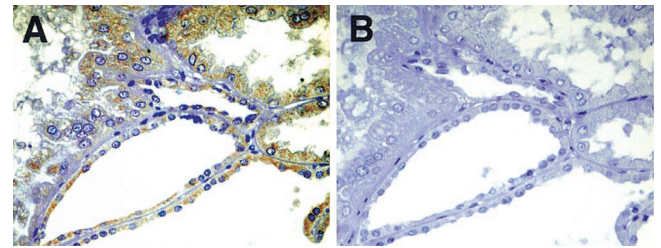


Fig. 3. (A) Immunohistochemistry for VEGF is positive in the epithelial cyst cells (brown color, Nikon, $\times 400$). (B) Negative control (Nikon, $\times 400$).

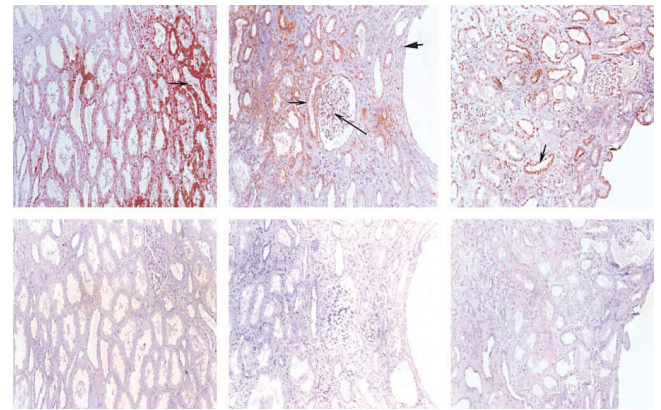


Fig. 4. Immunostaining for VEGF, VEGFR-2, and VEGFR-1 (left, center, and right upper panels, respectively) in areas of relatively preserved morphology. Glomeruli and remnant tubules are recognized. (Upper left) Staining for VEGF seen in some tubule cells (arrow). (Center) Staining for VEGFR-2 seen in interstitial areas with increased matrix, at high power corresponding to vasculature (short arrow, at the right), in some cells of the remnant glomerulus cells (long arrow) and in remnant tubule cells (narrow, short arrow at the left). (Right) Staining for VEGFR-1 seen in remnant tubule cells (arrow). No staining of interstitial structures or of the glomerulus (in the right upper corner) is seen. Lower panels are the negative controls. Left for VEGF, center for VEGFR-2, and right for VEGFR-1 (Nikon, $\times 100$).

VEGF is expressed in cyst cells

Vascular endothelial growth factor was demonstrated in cyst cells by immunohistochemistry. Expression levels were variable but observed in the cells of both fully developed cysts and small tubular dilations (Fig. 3A). The negative control is shown in Figure 3B. Image analysis by pixel value in a scale of 0 to 255 (**Methods** section) was 104 ± 2 in cytosol areas of cyst cells ($N = 185$, range 51 to 195) versus 51 ± 2 ($N = 63$, 19 to 85) in the interstitium ($P < 0.0001$). The negative control pixel value was 36 ± 2 for cyst cells ($N = 127$, $P < 0.0001$, positive cyst cells vs. cyst cells negative control) and 33 ± 2 for interstitium ($N = 127$, $P < 0.0001$ interstitium positive vs. negative control). Thus, the antibody recognized an epitope in the cyst cells as well as in some areas of the interstitium, although at lower levels. Figure 4 (left upper panel) shows staining for VEGF in intervening areas of

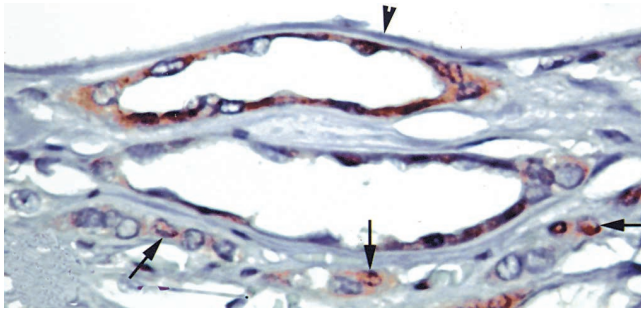


Fig. 5. Immunostaining demonstrates the expression of VEGFR-2 (KDR) in small vessels in the interstitium of a cyst. The narrow cyst epithelial cells (arrow at the top) do not express VEGFR-2 (Nikon $\times 400$).

more preserved morphology, demonstrating expression of VEGF protein in the cells of some enlarged tubules. A glomerulus on the field was not stained. A negative control is shown in the lower panel.

VEGF-receptors 1 and 2 expression in ADPKD kidney

In adult kidney, expression of VEGF-receptors 1 and 2 (VEGFR-1 and VEGFR-2) has been reported in glomeruli endothelial cells by in situ hybridization [18]. In tumors, it has been reported in endothelial and in cancer cells [17]. Immunostaining for VEGFR-1 and VEGFR-2 was positive in ADPKD. Staining for VEGFR-1 was found in the cells of small cystic tubules and in remnant tubules, as shown in Fig. 4 (upper right panel); no staining was found in the glomeruli or interstitial vasculature. Image analysis showed a pixel value (scale 0 to 255) of 101 ± 2 (range 30 to 221) in cystic tubules and tubule cells ($N = 292$), 38 ± 1 in the interstitium ($N = 155$, 4 to 85, $P < 0.0001$ cyst vs. interstitium), and 31 ± 2 in endothelial cells ($N = 52$, 9 to 72, $P < 0.0001$ cyst cells vs. endothelial cells). The negative control pixel values were 32 ± 2 ($N = 100$), 35 ± 2 ($N = 102$), and 33 ± 2 ($N = 50$) for cyst cells, interstitium, and endothelial cells, respectively (**Discussion** section).

Vascular endothelial growth factor receptor-2 (also known as KDR) was expressed in endothelial cells of small vessels surrounding cysts (Fig. 5), as well as in some glomerular cells and remnant tubules (Fig. 4, central upper panel). The negative control is shown in Figure 4 (lower-central panel). Image analysis showed a pixel value (scale 0 to 255) of 145 ± 4 for endothelial cells ($N = 119$, range 46 to 224) versus 32 ± 2 for interstitium ($N = 62$, 0 to 66, $P < 0.0001$), and 40 ± 3 for cyst cells ($N = 98$, 0 to 99, $P < 0.0001$ cyst vs. endothelial cells, $P < 0.0001$ cyst cells vs. interstitium). The negative control pixel values were 38 ± 3 ($N = 98$), 33 ± 2 ($N = 105$), and 33 ± 3 ($N = 50$) for cyst cells, interstitium, and endothelial cells, respectively.

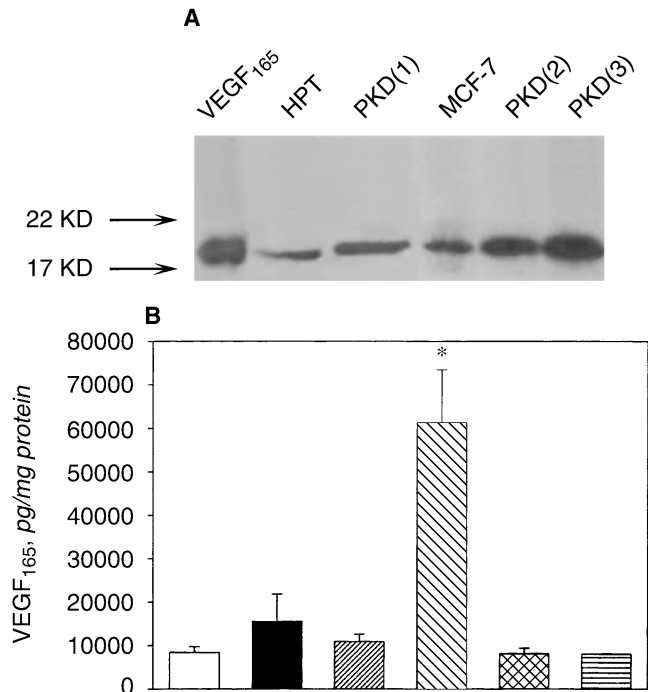


Fig. 6. (A) Determination of VEGF₁₆₅ expression by Western blot analysis with the specific monoclonal antibody VEGFab-4. Lane 1, control recombinant VEGF₁₆₅. Lane 2, protein from human proximal tubule cells in culture (HPT). Lanes 3, 5, and 6, protein from three different ADPKD cyst cell cultures: PKD(1), PKD(2), and PKD(3). Lane 4, protein from MCF-7 cells (a human cancer cell line). (B) VEGF₁₆₅ in pg/mg protein in the conditioned medium of ADPKD cyst cells cultured in 20% or 5% O₂ for 8 or 72 hours (**Methods** section). The release of VEGF₁₆₅ increased after incubation in low oxygen for 72 hours. Symbols are: (□) ADPKD, 20% O₂ for 8 hours; (■) ADPKD, 5% O₂ for 8 hours; (▨) ADPKD, 20% O₂ for 72 hours; (▩) ADPKD 5% O₂ for 72 hours; (⊗) MCF/7, 20% O₂ for 8 hours; (▧) MCF/7, 5% O₂ for 8 hours. * $P = 0.014$, $N = 6$.

ADPKD cell cultures produce soluble VEGF

Western blot analysis. VEGF-soluble protein was detected as a single band of approximately 19 kD. Bands shown in lane 1 of Figure 6A correspond to the positive control (recombinant VEGF₁₆₅); lanes 3, 5, and 6 correspond to three different isolates of protein from ADPKD cells; and bands in lane 4 loaded with lysate from MCF-7 cells (a cancer cell line). A lower expression was found in lane 2 corresponding to cultured human proximal tubule cells. Densitometric analysis of the specific bands showed that the relative expression of VEGF normalized to the recombinant VEGF₁₆₅ control was 30.5 ± 10.3 for proximal tubule (PT) cells, 82.4 ± 11.5 for ADPKD cells, and 56.2 ± 11.5 for MCF-7 cells ($N = 3$).

ELISA test demonstrates soluble VEGF in the culture medium from ADPKD cells. To confirm the up-regulation of VEGF by hypoxia, the amount of VEGF released to the culture medium was measured by the ELISA method. VEGF release was significantly higher in the medium from cells hypoxic for 72 hours ($10,879 \pm 1745$

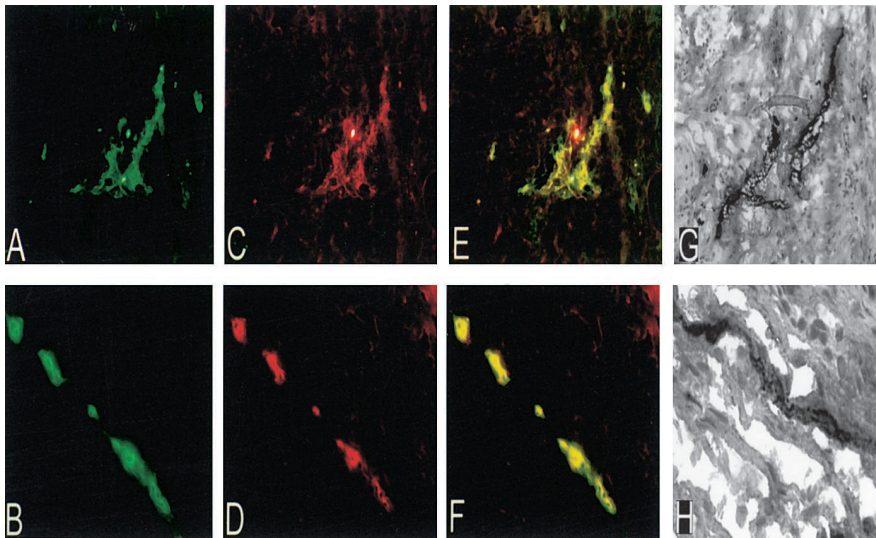


Fig. 7. (A and B) Immunofluorescence photomicrographs of pericycystic interstitial capillaries stained with FITC-anti-integrin $\alpha_v\beta_3$ antibody (green). (C and D) RITC-anti-MMP-2 immunostaining (red). (E and F) Colocalization of FITC-anti-integrin $\alpha_v\beta_3$ and anti-MMP-2 antibodies (yellow), indicating the coexpression in the lining of endothelial cells. (G and H) The corresponding fields to A and B (sections are serial, a few micrometers away) identifying the capillaries with antibody directed against Factor VIII-related antigen.

and $61,1321 \pm 12,145$ pg/mg protein for control and hypoxia, respectively, $N = 6$, $P = 0.014$). VEGF was also detected in the medium from MCF-7 cells (a breast cancer cell line) in amounts not different from those measured in the supernatant from ADPKD cells (8163 ± 1235 pg/8 h/mg protein, $N = 3$; $P = \text{NS}$; Fig. 6B). VEGF₁₆₅ was undetectable in the unconditioned media (data not shown).

Metalloproteinase-2 and integrin $\alpha_v\beta_3$ colocalize in kidneys from ADPKD. The expression of the vitronectin receptor integrin $\alpha_v\beta_3$ and MMP-2 were studied in ADPKD kidney sections. Both MMP-2 and integrin $\alpha_v\beta_3$ were functionally associated in the surface of angiogenic blood vessels [27]. It is believed that this cooperative interaction facilitates the invasive behavior of endothelial cells [27]. We found that there was coexpression of MMP-2 and integrin $\alpha_v\beta_3$ in the vessels of polycystic kidneys, in areas around the cysts, and in the expanded matrix (Fig. 7).²

DISCUSSION

Our study examined the vasculature of kidneys removed from patients with ADPKD and observed a rich network of vessels in the cyst walls. Although the renal circulation appears reduced in the angiographs, because of the fluid accumulated in the cysts and to the decrease in the mass of functional tissue, the cyst wall has a well-developed vascular network. Angiograms with radiopaque medium and with fluorescein indicate that the

vasculature is prominent in the cyst walls and that the vessels have an abnormal appearance (see ¹ on p. 40). Staining for factor VIII-related antigen demonstrated dilated vessels of abnormal morphology that in some places assume angiomatous configuration and extend into the *lamina propria* of the papilliferous grows into the cysts. Spiral and dilated vessels were observed in areas without evidence of inflammation but with interstitial fibrosis, indicating that the abnormalities of the vasculature are not limited to the areas of the cyst expansion. There is no other possible explanation for the extension of vasculature into the papilliferous growths that neof ormation of capillaries.

The VEGF and VEGFR-2 (KDR) in the normal adult kidney are expressed in the visceral epithelial cells and in the endothelia of glomeruli, respectively. The adult kidney also exhibits a weak expression of VEGF in the collecting duct and of VEGFR-2 in the surrounding capillaries [18]. The pattern of expression in ADPKD kidney was quite different: The epithelial cells of some cysts and enlarged tubules in more preserved areas express VEGF, and some capillaries around the cysts express VEGFR-2 (KDR) receptor, the receptor involved in neovascularization [28]. Expression of VEGFR-2 in some glomeruli was noted. The significance of the irregular staining of some cyst cells and in some remnant tubules for VEGFR-1 is unclear. The anti-VEGFR-1 antibody is believed to be a specific monoclonal antibody of human VEGFR-1. It reacts specifically with VEGFR-1 of human origin and does not cross-react with VEGFR-2 or other tyrosine kinases. The monoclonal anti-VEGFR-2 is derived from a KDR-2 hybridoma; purified recombinant human extracellular VEGFR-2 was the immunogen. The antibody is considered specific for human VEGFR-2. It does not recognize VEGFR-1 or other

²Sections were believed to be from normal areas from two kidneys removed because of cancer showed staining for MMP-2 and integrin $\alpha_v\beta_3$, but no areas of colocalization were found. The influence of soluble factors from the cancer cells and their vasculature in the integrin $\alpha_v\beta_3$ and MMP-2 expression could not be determined.

VEGF receptors or platelet-derived growth factor receptor. Thus, the reactivity found in cyst cells could represent a true abnormality of the ADPKD cells that will require further investigation, like the expression of a related protein. Expression of both receptors has been described in nonvascular cells of neural cells of the hypoxic retina, a finding taken to suggest an autocrine role of these receptors in the effect of VEGF [29]. Taken together, these experiments demonstrate that the conditions necessary to stimulate angiogenesis in the vicinity of the cysts are present in ADPKD kidneys.³

Vascular endothelial growth factor is also known as vascular permeability factor (VPF) and is recognized as potent agent—some 50,000 times more potent than histamine—that increases capillary permeability [30, 31]. Our findings suggest that cyst development is associated with local enhancement of the vasculature, which in turn may facilitate or promote fluid secretion into the cysts. Furthermore, we propose that the molecular mechanism of this process involve secretion of VEGF-stimulating neovascularization that would allow for the growth of the cyst, as a tumor, with a balanced blood supply. This process necessarily should differ from the neovascularization of malignancy, and could be triggered by hypoxia of the tubule cells and of the cysts as they expand. The process is unlikely to be synchronized to the whole kidney but would be present—at a given time—only in areas of active cyst growth.

Alternative gene splicing of a single VEGF gene can generate four different molecular species, of which VEGF₁₆₅ is the predominant form found in a variety of normal and transformed cells. VEGF₁₆₅ is secreted but a fraction remains bound to cell membrane or to the extracellular matrix, from where it can be released by heparin [reviewed in 17, 28]. Additional experiments were performed to determine whether a soluble form of VEGF was present in ADPKD cells grown in culture and if the soluble form was secreted to the culture medium. Our Western blot experiments and ELISA determinations both demonstrate VEGF₁₆₅ in ADPKD cells and supernatant (conditioned medium) of ADPKD cell cultures. We also found that VEGF secretion increased during hypoxia, one of the conditions that has been reported to up-regulate VEGF expression caused by transcriptional activation and increased mRNA stability [28]. Accordingly, we submit that the cyst cells produce VEGF that is secreted into the interstitium and is available to bind to the VEGFR-2 present in endothelial cells and

induces them to proliferate (**Methods** section). Evidence of the importance of this VEGF and VEGFR-2 interaction to induce the VEGF biological response is abundant in the literature, as well as evidence for VEGF's role in pathological angiogenesis [reviewed in 28]. In this context, our findings demonstrate the presence of a permissive condition for vascular remodeling and proliferation.

The cellular invasion by endothelial cells—be they in cancerous growths, inflammatory processes, or during repair—requires cell adhesion and migration mediated by integrins and matrix-degrading enzymes. In particular, the MMP-2 and the adhesion protein integrin $\alpha_v\beta_3$ have been shown to colocalize to the surface of invasive endothelial cells [32]. Our studies provide evidence that integrin $\alpha_v\beta_3$ localizes with MMP-2 on the endothelial surface of ADPKD blood vessels, a finding that supports the idea that both cell migration and the matrix degradation necessary for invasion by the neovasculature occur in ADPKD. MMP-2 has been demonstrated by immunohistochemistry in the septa between cysts and associated interstitium in kidneys of the C-57BL/6 J-cpk mice, a mouse model for autosomal recessive polycystic kidney disease (ARPKD). Thus, MMP-2 could act as a facilitator for the degradation of the interstitial matrix, allowing for cyst expansion [33].

Recently, it has been reported that mouse homozygous for Pkd1(L) mutant allele exhibit vascular fragility and were not viable after day 15.5, suggesting that polycystin-1 may have a functional role in maintaining vascular integrity during angiogenesis [34]. The relevance of this finding to the development of vascular abnormalities in ADPKD is yet to be determined.

In summary, the cysts in ADPKD are well vascularized, the vessels exhibit several malformations including aneurysmal and spiral forms, that the cyst cells express VEGF, and cyst cells in culture produce the soluble form. The VEGFR-2 (KDR) is present in some of the vessels of ADPKD, and there is colocalization of MMP-2 and integrin $\alpha_v\beta_3$. All of these findings indicate that the angiogenesis process occurs in ADPKD. The presence of VEGF, also known as VPF, may be important in increasing capillary permeability and favoring fluid transport into the cysts. In addition, inhibition of angiogenesis may open new avenues for treating the disease [11, 17, 28]. As a whole, our findings can be taken as complementary to the pathological changes, recognized as being of a tumorous nature, occurring in epithelial cells and culminating in cyst formation and expansion [35–37].

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³Gene expression of VEGF was detected in cyst cells (Genechip® Easi™; Affymatrix Inc. Santa Clara, CA, USA). A comparison of the luminescence signal with proximal tubule cells from normal kidneys did not show any significant difference. The cyst cells were negative for VEGFR-1 and VEGFR-2 gene expression, suggesting that the staining of tubule cells and cysts could represent the expression of another protein with the same epitope or be spurious.

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REFERENCES

- GABOW PA: Autosomal-dominant polycystic kidney disease. *N Engl J Med* 1993;329-332-342, 1993
- GABOW PA, GRANTHAM JJ: Polycystic kidney disease, in *Diseases of the Kidney*, edited by SCHRIER RW, GOTTSCHALK CW, Boston, Little Brown, 1997, pp 521-560
- WELLING LW, GRANTHAM JJ: Cystic disease of the kidney, in *Renal Pathology*, edited by Tisher CC, Brenner BM, Philadelphia, JB Lippincott Company, 1996, pp 1828-1863
- ITTER SA, BAEHR G: The arterial supply of the congenital polycystic kidney and its relation to the clinical picture. *J Urol* 21:583-592, 1929
- CORNELL SH: Angiography in polycystic disease of the kidneys. *J Urol* 103:24-26, 1970
- ETTINGER A, KHAHN PC, WISE HM: The importance of selective renal angiography in the diagnosis of polycystic kidney disease. *J Urol* 102:156-161, 1969
- MEANEY TF, CORVALAN JG: Angiographic diagnosis of polycystic renal disease. *Cleve Clin Q* 35:79-84, 1968
- WATSON ML, MACNICOL AM, ALLAN PL, WRIGHT AF: Effects of angiotensin converting enzyme inhibition in adult polycystic kidney disease. *Kidney Int* 41:206-210, 1992
- GUISTI R, NERI D, ANGELINI D, et al: Plasma concentration of endothelin and arterial pressure in patients with ADPKD, in *Autosomal Dominant Polycystic Kidney Disease: Contributions to Nephrology* (vol. 115), edited by SESSA A, CONTE F, SERBELLONI P, MILANI S, Basel, Karger, 1995, pp 118-121
- GRAHAM PC, LINDOP GBM: The anatomy of the renin-secreting cell in adult polycystic kidney disease. *Kidney Int* 11:1084-1090, 1988
- FOLKMAN J: Seminars in medicine of the Beth Israel Hospital, Boston: Clinical applications of research on angiogenesis. *N Engl J Med* 333:1757-1763, 1995
- AUGUSTIN HG: Antiangiogenic tumour therapy: Will it work? *Trends Pharmacol Sci* 19:216-222, 1998
- KLAGSBURN M, MOSES MA: Molecular angiogenesis. *Chem Biol* 6:R217-R224, 1999
- CARMELIET P, FERREIRA V, BREIER G, et al: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435-439, 1996
- LOSORDO D, VALE P, SYMES JF, et al: Gene therapy for myocardial angiogenesis: Initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischaemia. *Circulation* 98:2800-2804, 1998
- BAUMMGARTER I, OIECZEK A, MANOR O, et al: Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 97:1114-1123, 1998
- FERRARA N, DAVIS-SMYTH T: The biology of vascular endothelial growth factor. *Endocrinol Rev* 18:4-25, 1997
- SIMON M, GRONE HJ, JOHREN J, et al: Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *Am J Physiol* 268:F240-F250, 1995
- RUSS JC: *The Image Processing Handbook* (2nd ed). Boca Raton, CRC Press, 1995
- MIZE RR: Quantitative image analysis for immunocytochemistry and in situ hybridization. *J Neurosci Methods* 54:219-237, 1994
- HANAOKA K, DEVUYST O, SCHWIEBERT EM, et al: A role for CFTR in human autosomal-dominant polycystic kidney disease. *Am J Physiol* 270:C389-C399, 1996
- BELLO-REUSS E, WEBER MR: Electrophysiological studies on primary cultures of proximal tubule cells. *Am J Physiol* 251:490-498, 1986
- MATTHEWS DE, FAREWELL VT: *Using and Understanding Medical Statistics* (2nd ed). Basel, S. Karger, 1988
- TROCHA SD, KEVIL CG, MANCINI MC, ALEXANDER JS: Organ preservation solutions increase endothelial permeability and promote loss of junctional proteins. *Ann Surg* 230:105-113, 1999
- TOLENTINO MJ, ADAMIS AP: Angiogenic factors in the development of diabetic iris neovascularization and retinopathy. *Int Ophthalmol Clin* 38:77-94, 1998
- DWORKIN LD, BRENNER BM: The renal circulation, in *Brenner & Rector's The Kidney* (5th ed), edited by BRENNER BM, Philadelphia, W.B. Saunders, 1996, pp 247-285
- BROOKS PC, SILLETTI S, VON SCHALCHA TL, et al: Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase-2 fragment with integrin binding activity. *Cell* 92:391-400, 1998
- FERRARA N: Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 56:794-814, 1999
- SUZUMA K, TAKAGI H, OTANI A, et al: Increased expression of KDR/Flk-1 (VEGFR-2) in murine model of ischemia-induced retinal neovascularization. *Microvasc Res* 56:182-191, 1998
- SENGER SR, GALLI SJ, DVORAK AM, et al: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascitis fluid. *Science* 219:983-985, 1983
- SENGER DR, CONNOLLY DT, VAN DE WATER L, et al: Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. *Cancer Res* 50:1774-1778, 1990
- BROOKS PV, STROMBLAD S, SANDERS LC, et al: Localization of matrix metalloproteinase-2 MMP-2 to the surface of invasive cells by interaction with integrin $\alpha\beta 3$. *Cell* 85:683-693, 1996
- RANKIN CA, ITOH Y, TIAN C, et al: Matrix metalloproteinase-2 in a murine model of Infantile-type polycystic kidney disease. *J Am Soc Nephrol* 10:210-217, 1999
- KIM K, DRUMMOND I, IBRAGHIMOV-BESKROVNAYA O, et al: Polycystin 1 is required for the structural integrity of blood vessels. *Proc Natl Acad Sci USA* 97:1721-1736, 2000
- WOO DD, MIAO SY, PELAYO JC, WOOLF AS: Taxol inhibits progression of congenital polycystic kidney disease. *Nature* 368:750-753, 1994
- SWEENEY WE, CHEN Y, NAKANISHI K, et al: Treatment of polycystic kidney disease with a novel tyrosine kinase inhibitor. *Kidney Int* 56:33-40, 2000
- GRANTHAM JJ: Time to treat polycystic kidney diseases like the neoplastic disorders that they are. *Kidney Int* 57:339-340, 2000